



Neural Differentiation and Developmental Effects of Honey Bee Venom on PC12 Cells; A Comparison to Nerve Growth Factor (NGF)

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Abstract: The rat pheochromocytoma cell line, PC12 that in *in-vitro* condition under inductive factors differentiate and convert into neuron-like cells. Researches have shown that different factors, for example, NGF and bee venom or its components have different effects on proliferation, survival and differentiation of the cells. In this study the PC12 cells were seeded in the culture medium (RPMI-1640) at 5×10^3 cell/well in poly-D-lysine (0.05mg/ml) coated 24- well culture plates, allowed to grow for 24hrs, and PC12 cells were treated with NGF for 10 days and were surveyed morphologically in the 1st, 3rd, 5th and 7th days and used various concentrations of honey bee venom for cellular differentiation analysis that the optimum concentrations were 1 μ g/ml and 3 μ g/ml, and using NGF with the concentration of 50ng/ml and the venom with the concentrations of 1 μ g/ml and 3 μ g/ml simultaneously. The viability of PC12 cells was analyzed by using MTT assay, and cell differentiation were surveyed AChE activity assay and morphologically.

Keywords: Bee venom, PC12 cells, Differentiation, NGF, Neuron.

1. Introduction

Neurotrophic factors regulate survival and differentiation of neurons in the peripheral and central nervous systems. These factors include fibroblast growth factors (FGFs), the interleukin-like factor ciliary neurotrophic factor and the neurotrophin family. The neurotrophin family of factors includes nerve growth factor (NGF), neurotrophin-3 (NT-3), brain derived neurotrophic factor (BDNF) and NT-4/5. Neurotrophins expedite the differentiation and survival populations of neurons in culture and show specific temporal and distinct expression patterns. Neurotrophins link to the Trk family of receptor tyrosine kinases, which includes Trk, TrkB and TrkC. NGF links to Trk; BDNF, NT-3 and NT-4/5 link to TrkB; and NT-3 links to TrkC. NT-3 and NT-4/5 can also bind weakly to Trk (1, 3, 9 and 14). NGF is the best-described neurotrophic factor. NGF is necessary for differentiation and survival of

sympathetic and some sensory neurons and for the cholinergic neurons. NGF is one of the factors that further differentiation of the rat pheochromocytoma tumor cell line PC12 (5). The PC12 cells are one of the best models for investigation of cellular differentiation induced by neurotrophic factors. When PC12 cells were exposed with NGF or FGF for several days some of events occur, including extension of neurites and appear of a differentiated phenotype patterned by the development of Excitability electricity and produce of neurotransmitters (7, 11, 13 and 22). Predecessor observations have shown that bee venom or its components are effective in proliferation, survival and differentiation of the cells. The most important component that effects in the differentiation of PC12 cell line is PLA2 (phospholipase A2). PLA2 acts as neurotrophin-like factor in the rat pheochromocytoma PC12 cell line. Observations have shown that PLA2 decreases the cells from apoptosis (15 and 16).

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One of the first cell types that attended to have the discriminatory features of a neuroendocrine cell was adrenal chromaffin cell. Chromaffin cells have been vastly utilized as a model for studying the manners of secretion and hormone extricate from neuroendocrine cells. Researches show that since these cells realize catecholamines, especially dopamine, transplantation of these cells to the substantia nigra has been noticeable repair dopaminergic neuronal deficit in Parkinson's disease (1). If PC12 cell exposure to 50ng/ml NGF for several days, these cells will proliferate and extension of structures morphologically similar to primary sympathetic neurons. Because of this trait, PC12 cells supply a great experimental model system to survey collective effects in neuritogenesis (5).

For assessing differentiation of PC12 cells often used semiquantitative or quantitative morphological methods. These methods including determination of cell size, the number of cells exhibiting processes (neurites) and the extent of neurite growth or neurite.

2. Materials and Methods

PC12 cells were obtained from the Iran Pasteur Institute, Tehran. The Iranian Honey Bee (*Apis mellifera*) Venom (BV) was prepared by placing bee on a 6-mm wire grid, which was electrically pulsed. The bees then produced venom that dropped to a glass plate beneath, which was collected from the glass and freeze-dried, according to the method of Lariviere (5).

2.1 Cell culture

PC12 cells were obtained from a Pastor institution in Iran and maintained in RPMI 1640 (Sigma) containing 10% fetal bovine serum (FBS, Sigma) supplemented with 100 U/ml penicillin, 100g/ml streptomycin, at 37°C in a humidified atmosphere, 5% CO₂. Cells were ordinary subcultured every 3 days. For differentiation, cells were plated at 5×10^3 cells/well in poly-D-lysine (0.25mg/ml in double distilled water) - coated 24-well plates and allowed to adhere for 24 h in RPMI medium plus 10% bovine serum. After 24 h medium was changed to RPMI plus 1% FBS and stimulating factors. The concentrations used were 50ng/ml for NGF and 1 and 3µg/ml for Bee venom (2, 4, 5 and 6).

2.2 Dose-Response Analysis

BV preparations at concentrations of 1, 2, 3, 5, 7 and 10µg/ml were added to each overnight cultured cell. Non-treated cells were used as controls. Cells were cultured overnight and then subjected to MTT assay.

2.3 Differentiation and quantitative morphology

Morphometric analysis was performed by inverted microscope linked to a black and white camera. Images of five fields per well were taken with an average of 10 cells per field. The number of differentiated cells was

determined by visual examination of the field and counting cells that had at least one neurite with a length equal to the cell body diameter and expressed as a percentage of the total cells in the field. Neurite growth was determined by manually tracing the length of the longest neurite per cell (using Image J software) for all cells in a field that had an identifiable neurite and for which the entire neurite arbor could be visualized. Data from the five fields in each well were pooled, and each well was designated as a "n" of one. The experiments were repeated at least three times using cultures prepared on separate days (5, 10, 9, 18 and 21).

2.4 Cell proliferation assay

PC12 cells were cultured on 96-well plates (10³ cell/well) in complete RPMI 1640 media for 24 h, then treated with or without 50ng/ml NGF and 1, 3 and 5µg/ml Bee venom for 2 to 8 days and the culturing media were refreshed every 3 days. MTT assays were performed on days 1, 3, 5 and 7. At a fixed time on each day, 10µl MTT (5mg/ml) was added to each well containing 100µl culture media and was carefully mixed. The mixtures were incubated at 37°C for 4 hr, and 100µl isopropyl alcohol (containing 0.04mM HCl) was then added and incubated for 5 hr. The optical absorbance was determined at 570nm with ELISA reader (8, 10 and 12).

2.5 AChE activity assay

AChE activity was measured by a standard spectrophotometric method. The activity specifically attributable to acetylcholinesterase was determined using DTNB (dithionitrobenzoic acid, Sigma, USA). The cells were removed from the tissue culture dishes by the addition of PBS containing trypsin and EDTA (17, 20 and 19).

2.6 Statistical analysis

Data have been presented as the mean \pm standard error of the mean (SEM). Statistical comparisons were made by analysis of variance (ANOVA) and when significant differences were observed, Tukey's test was employed for multiple comparisons. Statistical significance was inferred at $P < 0.05$.

3. Results

3.1 The determination of appropriate dose of honey bee venom for PC12 cell line differentiation towards neuron

The concentration of bee venom which causes the death of 50% of cells, was considered as LD₅₀. In this experiment, the concentration of 5mg/ml of bee venom was measured as the concentration of LD₅₀. So, the concentrations lower than 5mg/ml such as 1mg/ml, 2mg/ml and 3mg/ml were used in order to differentiate between cells.

3.1.1 The analysis of PC12 cell line differentiation percent to distinguishable neurons using NGF with the concentration of 50ng/ml

In these experiments, PC12 cells were treated with NGF for 10 days and were surveyed morphologically in the 1st, 3rd, 5th and 7th days. The treated cells were morphologically similar to a controlled case on the first day and no differentiation were observed. The neurites analysis began to show as sprouts around the cells on the third day and them developed gradually, and cell differentiation were quite distinct morphologically on the fifth day and the neuritis extended like complicated networks and nearly more than 80% of PC12 cells differentiated to neurons and this differentiation reached the maximum level on the seventh day and almost all cells were differentiated and neuritis length were maximum (Fig. 1 A, B).

3.1.2 The analysis of PC12 cell line differentiation to distinguishable neurons using the honey bee venom with appropriate concentrations

We used various concentrations of honey bee venom for cellular differentiation analysis in our experiments and the optimum concentrations were 1µg/ml and 3µg/ml. The venom morphological analyses with the concentration of 1µg/ml showed that the number of distinguishable cells was low (nearly 8%) in the first day and there was a small increase in this number on the third day, but the number of distinguishable cells increased rapidly from this day on, so that this number reached its maximum level (25%)

on the fifth day and the cells began to die in the next days (Fig. 1C).

For the venom with the concentration of 3µg/ml, as compared with the one with the concentration of 1µg/ml, the number of distinguishable cells increased so that nearly 15% of cells in the first day and 25% of cells. On the third day were differentiated, and the number of distinguishable cells was maximum on the fifth day and nearly 38% of cells were differentiated in this day. The number of distinguishable cells was stable in the next days and cellular death was increased in such a way that almost all of the cells were perished on the seventh day (Fig. 1D).

3.1.3 The analysis of PC12 cellular differentiation to distinguishable neurons using the honey bee venom and NGF simultaneously

The NGF with the concentration of 50ng/ml and the venom with the concentration of 1µg/ml and 3µg/ml were used in these experiments. In general, simultaneous differentiation of PC12 cells was more than the one with the NGF and venom separately. There was a little difference between simultaneous differentiation and the one with the venom was remarkable. Also, the simultaneous differentiation of NGF with the venom with concentration of 3µg/ml was higher in comparison with the one with a concentration of 1µg/ml. However, the life of cells in the simultaneous process was nearly similar to the one with NGF and the life of treated cells in this process was longer than the one with the venom (Fig. 1E, F and see Table 1 and Fig. 2).

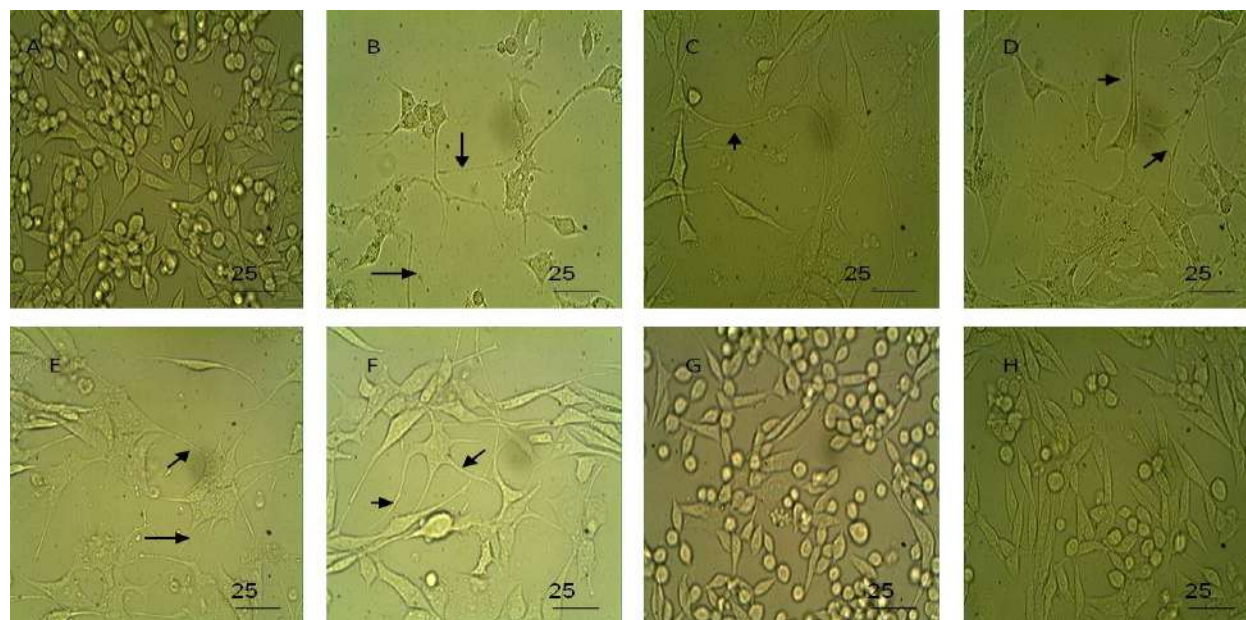


Fig. 1. PC12 cells with NGF treatment (50ng/ml) after 1 day. (A) PC12 cells with NGF treatment (50ng/ml) after 5 days. (B) PC12 cells with bee venom treatment (1µg/ml) after 5 days. (C) PC12 cells with bee venom treatment (3µg/ml) after 5 days. (D) PC12 cells with bee venom (1 µg/ml) and NGF (50ng/ml) treatment after 5 days. (E) PC12 cells with bee venom (3µg/ml) and NGF (50ng/ml) treatment after 5 days. (F) PC12 cells in the absence NGF or bee venom (control) after 1 day. (G) PC12 cells in the absence NGF or bee venom (control) after 5 days. (H) arrows show processes in differentiated cells. Mag. 400x.

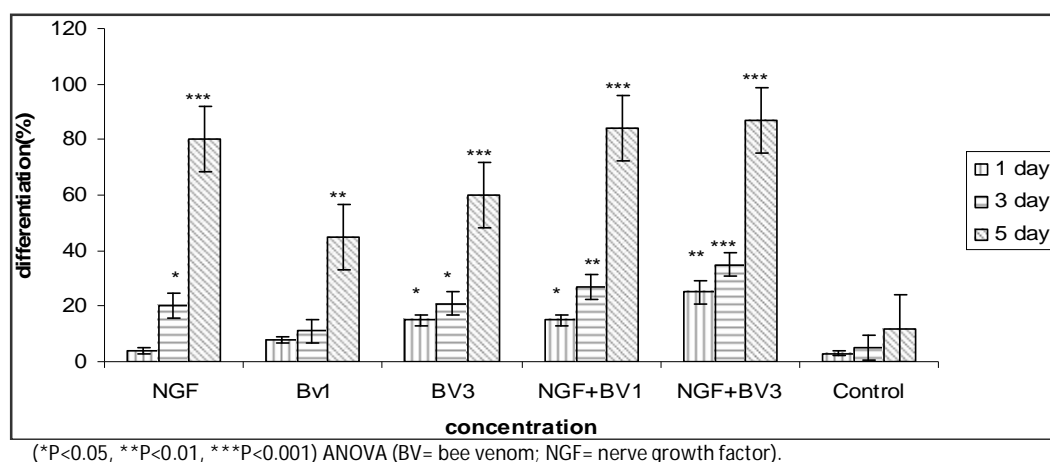


Fig. 2. Effect of NGF and bee venom on differentiation PC12 cell line by use.

Table 1. Effect of NGF and bee venom on differentiation PC12 cell line.

Differentiated neurons number				Experimental groups
Day 7	Day 5	Day 3	Day 1	
85±2.000	80±1.000	20 ± 2.000	4±1.000	NGF
0±0.000	25±2.000	11 ± 1.000	8±1.000	BV1
0±0.000	38±5.000	21 ± 1.000	15 ± 2.000	BV3
85±4.000	84±4.000	27 ± 4.000	15±2.000	NGF+BV1
88±6.000	87±2.000	35 ± 2.000	25 ± 4.000	NGF+BV3
0±0.000	12±1.000	5±1.000	3±1.000	Control

3.2 The analysis of the viability of the PC12 cell line using MTT process

3.2.1 The analysis of the viability of the treated PC12 cells using NGF with concentration of 50ng/ml

The NGF is one of the factors which cause the life and viability of cells. This factor was considered in our experiments, too. In the experiment on PC12 cells, the viability was 100% in the first and third day and it was 93% on the fifth day.

3.2.2 The analysis of the treated PC12 cells using the honey bee venom with various concentrations

The honey bee venom with concentrations of 1, 2, 3, 5 and 7µg/ml were used in this analysis. Among these concentrations, the concentration of 5µg/ml was determined as LD₅₀, because the cellular viability was 50% in the first day of analysis and almost all of the cells died in higher concentrations.

The cellular viability of venom with the concentration of 1µg/ml was higher in comparison with other concentrations, and the more the concentration of venom increases the less the cellular viability will be. The noticeable point in this experiment is that the cellular viability of the venom with concentrations of 1, 2 and 3µg/ml increases from the first day till the third day, and it decreases remarkably till the fifth day. In general, the viability of the treated cells using the

venom is much lower than the one using NGF, especially on the fifth day.

3.2.3 The analysis of the treated PC12 cells using NGF and the venom simultaneously

The NGF with the concentration of 50ng/ml and the venom with the concentrations of 1µg/ml and 3µg/ml were used in this experiment. The cellular viability in a simultaneous process in comparison with NGF and the venom was meaningfully lower and higher, respectively (See the results of viability using the MTT process in Fig. 3 and Table 2).

Table 2. Effect of NGF and bee venom on viability PC12 cell line.

Viability			Experimental groups
Day 5	Day 3	Day 1	
93 ± 3.000	100 ± 0.500	100 ± 0.500	NGF
15 ± 2.000	85 ± 2.000	75 ± 3.000	BV1
15 ± 1.000	79 ± 1.000	71 ± 3.000	BV2
10 ± 3.000	71 ± 3.000	62 ± 3.000	BV3
4 ± 1.000	47 ± 3.000	49.5 ± 1.500	BV5
53 ± 4.000	93 ± 3.000	92 ± 2.000	NGF+BV1
41 ± 4.000	88 ± 1.000	86 ± 3.000	NGF+BV2
37 ± 5.000	80 ± 5.000	83 ± 1.000	NGF+BV3
19±0.000	60±2.000	65±2.000	NGF+BV5
100±0.000	100±0.000	100±0.000	CONTROL

3.3 The analysis of enzyme reaction (AChE)

This analysis was conducted 12 days after treating of cells. The results of the experiments showed that differentiating cells by NGF and by the venom with the concentration of 3µg/ml and the simultaneous increase of them had enzyme actions which in the level with $P<0.05$ (for the honey bee venom) and the one with $P<0.01$ (for the NGF process alone and the simultaneous treating with NGF and the honey bee venom) showed a meaningful increase as compared with the control group, that were entirely matched with the obtained results from the analysis of morphological cells (The results were demonstrated in Table 3 and Fig. 4).

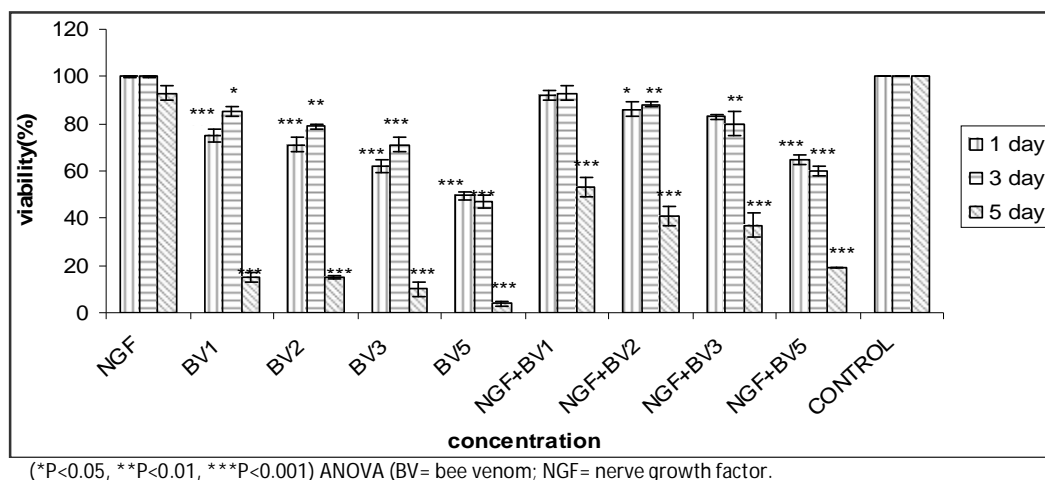


Fig. 3. Effect of NGF and bee venom on viability PC12 cell line by use.

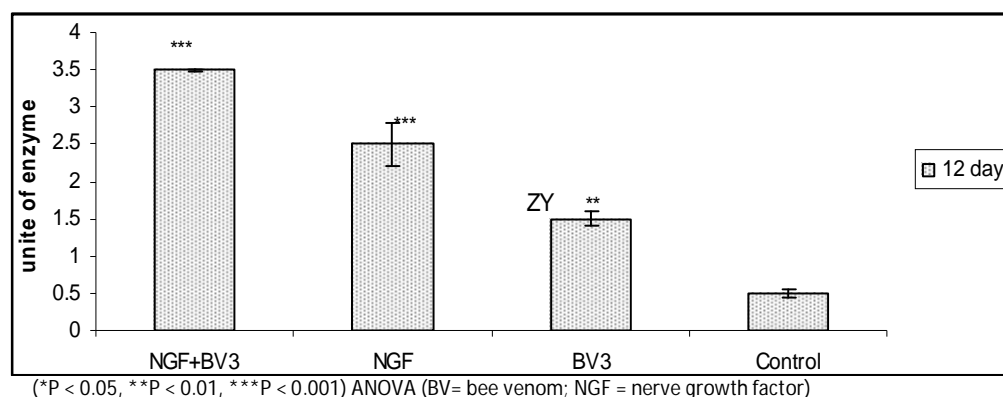


Fig. 4. Enzyme activity in PC12 cell line treated by NGF and bee venom after 12 days use.

Table 3. Enzyme activity in PC12 cell line treated with NGF and bee venom.

Experimental groups	Unit of enzyme (day 12)
control	.5 ± 0.05
NGF	2.5 ± 0.29
BV3	1.5 ± 0.09
NGF+BV3	3.5 ± 0.011

4. Discussion

PC12 cells were used in this experiment because of the ability to change them to the neural cells in laboratory conditions. And these cells were cultured in the presence of neuronal differentiation factors in order to develop neural cells. There is a belief that by achieving the technical knowledge of neural cells production and by providing the possibility of their differentiation, these cells can be used in the analysis of cell therapy and cellular graft.

In 2006, Ohnuma *et al.*, stated that PC12 cells were proliferated in appropriate culture medium, and the proliferation was ceased after treating with NGF, and

they differentiated to pseudo-sympatric neural cells. Also, our findings admit these results (17).

In 2004, Das *et al.*, explained that the PC12 cells were round and small, in the absence of NGF, and they had a little outgrowth. However, exposing PC12 cells to NGF during seven days increased thickness of these cells and the neuritis were developed, too. For the analysis of neurons differentiation, both the number of neurite cells and neurite length were measured in this study. Cells treating NGF on the sixth day by counting the number of distinguishable cells in our experiments, and nearly 95% of cells were differentiated. From the former times, bee therapy was used in curing many diseases such as infectious, inflammatory and articulate illnesses. Nowadays, it was also proved that the honey bee venom and its components had important effects on proliferation, life and growth of cells (4).

In 2003, Nakashima *et al.*, stated that phospholipase A2 induced neuritis in PC12 cells, and this activity caused to fatty acids. And even based on a number of fatty acids like Arachidonic acid, the sum of phospholipase A2 activities could be measured. The

researches showed that neurite-outgrowth activity with phospholipase A2 or its homologous like P15 was nearly depended on the ability of phospholipase A2 in freeing the fatty acids in PC12 live cells (16).

In 2005, Masuda *et al.*, admitted the results obtained by Nakashima. Besides, they examined both the simultaneous and separate differentiation effects of phospholipase A2 and NGF in their experiments. They stated that the separate use of phospholipase A2, as compared with the one using NGF, had less effect on neurite outgrowth in PC12 cells, and the simultaneous use of it with NGF caused to increase neurite-outgrowth in PC12 cells (15).

5. Conclusions

In our research, we used the honey bee venom completely and the number of neuritis cells in treated PC12 cell line using the honey bee venom with concentration of 1µg/ml was 15% on the fifth day of culture, and the number of neuritis cells with the concentration of 3µg/ml was 38% in the same day. While in Nakashima experiments, the number of neuritis cells in treated PC12 cell line using phospholipase A2 with concentration of 10 nanomolar was 37% on the fifth day.

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